

HID EVolution™ – qPCR/STR Setup System

SUBJECT: Quantifiler® Duo DNA Quantification Kit Support and Validation – New Scripts and Converter Software

Purpose of this user bulletin

The Quantifiler® Duo DNA Quantification Kit (Quantifiler® Duo kit) is now supported for automated qPCR reaction setup on the HID EVolution™ – qPCR/STR Setup System (qPCR/STR system). This user bulletin:

- Provides Quantifiler Duo kit-specific instructions to supplement the procedures in the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide* (PN 4426903).
- Describes the validation and verification studies of quantitative PCR (qPCR) setup on the qPCR/STR system using the new Quantifiler Duo scripts and the Quantifiler Duo Converter Software v1.0.

In this user bulletin

This user bulletin covers:

- Using the Quantifiler® Duo DNA Quantification Kit with the HID EVolution™ – qPCR/STR Setup System 2
- Validation of Quantifiler® Duo Kit on HID EVolution™ – qPCR/STR Setup System 16

Prerequisites

This user bulletin assumes that:

- You have access to the following documents:
 - *HID EVolution™ – qPCR/STR Setup System Getting Started Guide* (PN 4426903)
 - *Quantifiler® Duo DNA Quantification Kit User's Guide* (PN 4391294)
 - *Tecan HID EVolution™ - qPCR/STR Setup System Application Manual*
 - *Tecan Freedom EVO® Operating Manual*
- You have referred to the manufacturer's instrument documentation for important safety information related to the use of the Tecan Freedom EVO® instrument.

Section 1 Using the Quantifiler® Duo DNA Quantification Kit with the HID EVolution™ – qPCR/STR Setup System

This section covers:

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Workflow for the Quantifiler® Duo DNA Quantification Kit

To use the Quantifiler® Duo kit on the qPCR/STR system, follow the instructions in the *HID EVOLution™ – qPCR/STR Setup System Getting Started Guide* (PN 4426903) with the added and modified procedures listed in [Table 1](#) and explained on [pages 4](#) through [15](#) of this user bulletin.



Note: [Figure 1 on page 5](#) illustrates the files imported and exported throughout the automated HID workflow.

Table 1 Workflow for using the Quantifiler® Duo kit on the qPCR/STR system.

To perform this workflow step...	Follow the procedures in...
One-time tasks	
Perform one-time tasks	a. Download and install Quantifiler® Duo scripts and Converter software on page 4 .
	b. Create detectors for the 7500 Real-Time PCR System on page 5 .
	c. If you have not already done so, perform the other one-time tasks described in the Getting Started Guide (GSG) [‡] Chapter 2, “Pre-Run Procedures”.
qPCR reaction setup and qPCR	
1. Perform routine maintenance and other pre-run procedures	GSG Chapter 2, “Pre-Run Procedures”.
2. Prepare qPCR reagents and labware	a. Determine required reagent volumes on page 6 .
	b. Place reagents in the qPCR reagent block on page 8 .
	c. Set up the samples, reagents, and plasticware on the worktable on page 10 .
3. Run automated qPCR reaction setup	GSG Chapter 4, “Run Automated qPCR Setup,” except that you use the scripts on page 11 .
4. Perform qPCR and review results	a. Convert and transfer the 7500 setup file on page 11 .
	b. Import the converted 7500 setup file and perform qPCR [§] on page 12 .
	c. Analyze and review the qPCR results on page 13 .
	d. Export and convert the 7500 results files on page 14 .
STR PCR reaction setup	
1. Prepare STR PCR reagents and plasticware	GSG Chapter 6, “Prepare STR PCR Reagents and Labware”.
2. Run automated STR PCR reaction setup	GSG Chapter 7, “Run Automated STR PCR Setup” except that you use the instructions for importing qPCR results on page 15 .

[‡] *HID EVOLution™ – qPCR/STR Setup System Getting Started Guide* (PN 4426903).

[§] The converted 7500 setup file is imported as a SDS plate record.

One-time tasks

Download and install Quantifiler® Duo scripts and Converter software

- ❗ **IMPORTANT!** To use the Quantifiler® Duo kit on the qPCR/STR system, you must download and install the:
- HID EVolution™ - Quantifiler® Duo Converter Software v1.0
 - HID EVolution™ - Quantifiler® Duo Scripts

About the Quantifiler® Duo kit Converter Software

The HID EVolution™ - Quantifiler® Duo Converter Software ensures that the Quantifiler Duo assay sample data is handled correctly by the SDS and qPCR/STR system software. Use the Converter to:

- Convert the 7500 setup file before you import the file into the SDS software
- Convert the 7500 results file before you import the file(s) into the qPCR/STR system software

Figure 1 on page 5 illustrates the files imported and exported throughout the automated HID workflow. The Quantifiler Duo scripts, Converter software, and converted files are flagged with “**”.

Download and install software and scripts

1. Go to www.tecan.com/HID.
2. Click **Download** on the right side of the page under Product Details.
3. Download the Quantifiler® Duo scripts and Converter software.
4. Unzip the downloaded files, then follow the instructions included with the download to install the scripts and Converter.

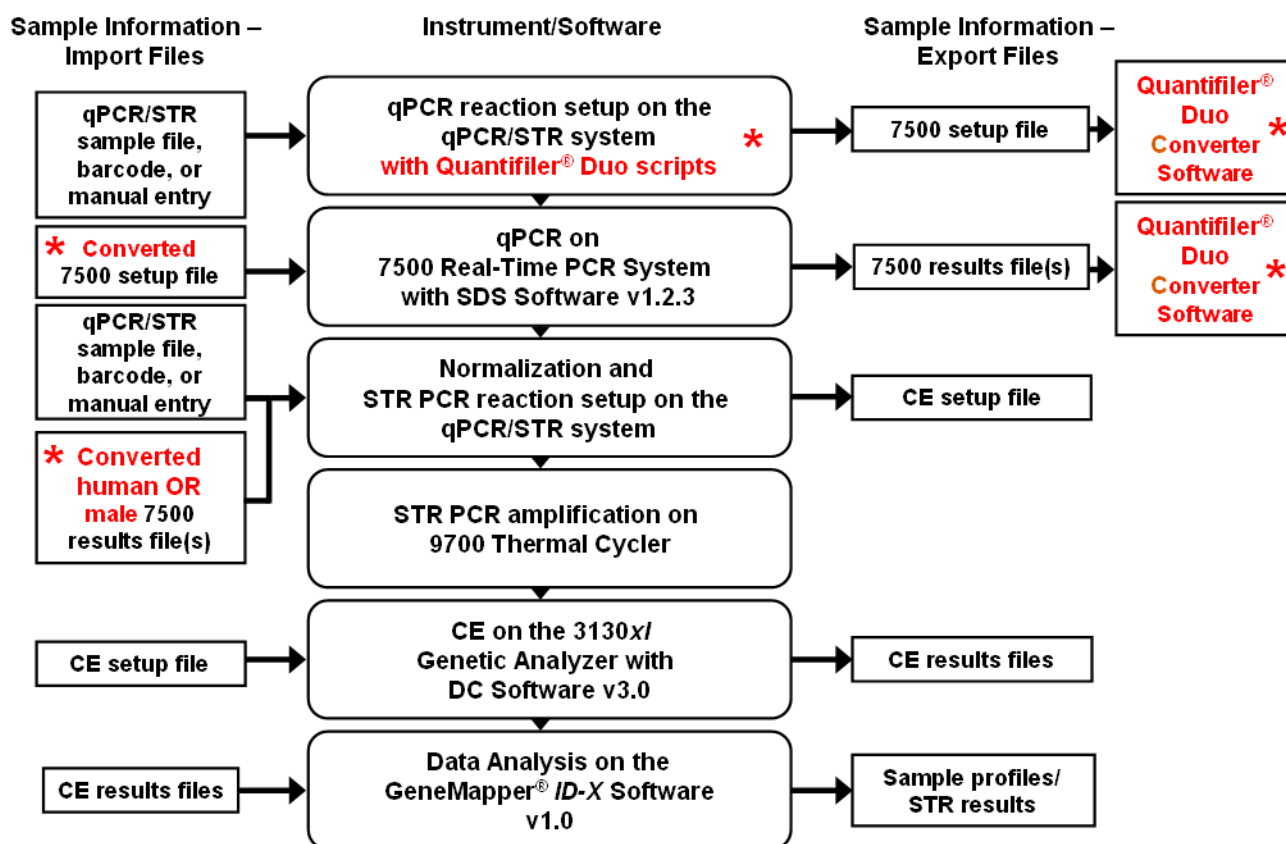


Note: The scripts must be installed on the qPCR/STR system computer. The Converter software is stand-alone software (not part of the Freedom EVOware® or SDS software). For ease of use, we recommend that you install the Converter software on the qPCR/STR system computer.



Note: The downloaded files also include instructions for using the Converter: *HID EVolution™ - qPCR/STR Setup System*, *HID EVolution™ - Combination System*, *Quantifiler® Duo Converter Manual* (Tecan Document ID 396042).

Figure 1 Automated HID workflow for the Quantifiler® Duo kit. The Quantifiler Duo scripts, Converter software, and converted files are shown in red text and marked with *.



Create detectors for the 7500 Real-Time PCR System

If you have not already done so, create the following detectors in the SDS Software v1.2.3 for running the Quantifiler® Duo kit assays. Refer to the Applied Biosystems *Quantifiler® Duo DNA Quantification Kit User's Manual* for details on creating the detectors.

IMPORTANT! The detectors must have the exact names and capitalization shown below.

Detector Name	Reporter Dye	Quencher Dye
Duo Human	VIC	Quencher Dye = (none) (make sure (none) is selected)
Duo Male	FAM	Quencher Dye = (none) (make sure (none) is selected)
Duo IPC	NED	Quencher Dye = (none) (make sure (none) is selected)



Note: These detectors allow you to import the *converted* qPCR/STR system-generated 7500 setup file to the SDS Software.

Prepare qPCR reagents and labware



Note: Perform routine maintenance before placing reagents, labware, and samples on the worktable. Refer to Chapter 2, “Pre-Run Procedures” in the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide*.

Determine required reagent volumes



Note: Applied Biosystems recommends that you combine reagents from different tubes from the same lot if necessary to meet the minimum volume requirements.

1. Determine the volume of DNA standard and dilution buffer (or volume of pre-prepared DNA standard dilution series) that you need. You can either:
 - **Let the qPCR/STR system prepare the DNA standard dilution series for you.** Use the table below to determine the required volume of undiluted Quantifiler® Duo DNA Standard and Quantifiler® Duo Dilution Buffer.

Required volumes for qPCR/STR system-prepared DNA standard dilution series

Reagent	Available volume in full tube of reagent	Minimum volume [‡] required on worktable
Undiluted Quantifiler® Duo DNA Standard	120 µL	75 µL
Quantifiler® Duo Dilution Buffer	1.8 mL	825 µL

[‡] Includes 50 µL dead volume per tube necessary to ensure that the pipette tips remain submerged during aspiration so that liquid, not air, enters the tips.

- **Use a pre-prepared DNA standard dilution series**, if the pre-prepared series meets the following three requirements:
 - The series was prepared in the last two weeks.
 - The series was prepared according to the procedures specified in the *Quantifiler® Duo DNA Quantification Kit User's Manual* or was prepared by the qPCR/STR system.
 - At least 30 µL of each of the eight concentrations of diluted standard is available. For reliable liquid detection, it is recommended to use at least 50 µL of each of the eight concentrations of diluted standard.



Note: Two replicates of the eight concentrations in the DNA standard dilution series are transferred to the qPCR reaction plate for a total of 16 DNA standard dilution series samples.

30 µL includes the 4 µL necessary to prepare the two replicates of each concentration in the DNA standard dilution series, plus the minimum required overfill volume.

2. Calculate the required volume of Quantifiler® Duo kit reagents using [Table 2](#).



IMPORTANT! To obtain the required volume of primer mix, you can place up to 3 tubes of primer mix in the reagent block for one run. If you divide the minimum required volume of primer mix across 2 or 3 tubes, each tube must contain a multiple of 190 µL *plus* an additional 50 µL per tube to ensure that the instrument detects adequate volume to prepare the selected number of reactions. Refer to “About minimum required reagent volumes” on page 25 of the *HID EVOLUTION™ – qPCR/STR Setup System Getting Started Guide*.



Note: The volumes in the table include the excess volume required per sample and per run necessary to compensate for evaporation and pipetting losses during the run.

Table 2 Reagent volumes for qPCR reaction setup with the Quantifiler® Duo DNA Quantification Kit (25-µL reaction volume)

Reagent	Available volume in full tube of reagent	Required volume per reaction	Extracted DNA samples per run	Diluted standard samples per run	Required excess reactions per run	Required dead volume† per tube	Minimum required volume for 80 extracted DNA samples and 16 diluted standards = $[A \times (B + C + D)] + E$
		A	B	C	D	E	
Quantifiler® Duo Primer Mix	1.4 mL	11.55 µL	up to 80	16	3	50 µL	1194§ µL in one tube#
Quantifiler® Duo PCR Reaction Mix	5 mL	13.75 µL	up to 80	16	3	50 µL	1412‡ µL in one tube

† A 50-µL dead volume per tube is necessary to ensure that the pipette tips remain submerged during aspiration so that liquid, not air, enters the tips.

§ $[11.55 \mu\text{L}/\text{reaction} \times (80 + 16 + 3 \text{ reactions})] + 50 \mu\text{L}/\text{tube} = 1193.45 \mu\text{L}$ in one tube.

If you divide the minimum volume of Primer Mix across multiple tubes, each tube must contain a multiple of 190 µL plus an additional 50 µL per tube to ensure that the instrument detects adequate volume to prepare the selected number of reactions. For example, place at least 1000 µL in the first tube and 240 µL in the second tube.

‡ $[13.75 \mu\text{L}/\text{reaction} \times (80 + 16 + 3 \text{ reactions})] + 50 \mu\text{L}/\text{tube} = 1411.25 \mu\text{L}$ in one tube.

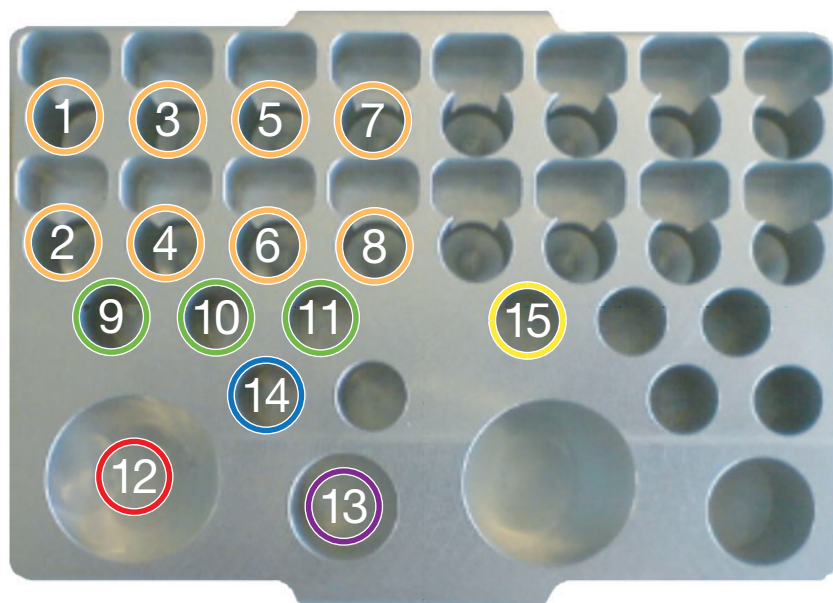
Place reagents in the qPCR reagent block

Use the following procedure to place the Quantifiler® Duo kit reagents into the correct positions in the chilled qPCR reagent block.

1. Thaw the reagents, then prepare according to the *Quantifiler® Duo DNA Quantification Kit User's Manual*:
 - Thaw the primer mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
 - Swirl the PCR reaction mix gently before using. Do not vortex it.
 - Vortex the DNA standard 3 to 5 seconds.
2. If you are using a previously-prepared DNA standard dilution series, vortex and briefly centrifuge the DNA standard dilutions at low speed before use.
3. Place the labeled empty tubes (or labeled pre-prepared DNA standard dilution series) in positions 1 through 8 as shown in [Table 3 on page 9](#).
 - ⓘ **IMPORTANT!** Make sure to place each concentration in the correct location in the reagent block, as shown in the orange table in [Table 3 on page 9](#).
4. Place the reagents in positions 9 through 15 as shown in [Table 3 on page 9](#).

Table 3 Reagent block configuration for Quantifiler® Duo kit

50.0	5.556	0.617	0.069
16.667	1.852	0.206	0.023



Legend:

1-8. 1.5-mL tubes of pre-prepared DNA standard dilution series (arrange concentrations as shown in the corresponding orange table) OR 1.5-mL empty tubes for DNA standard dilution series.

9-11. Up to three 1.5-mL tube(s) of Quantifiler® Duo Primer Mix. Place the first tube in position 9. If you use more than one tube, continue to position 10, then position 11.†

12. Quantifiler® Duo PCR Reaction mix.

13. Empty 5-mL VWR tube (VWR part number 890005-596) for the master mix.

14. (If the run includes preparation of DNA standard dilution series) Quantifiler® Duo DNA Standard tube.

15. (If the run includes preparation of DNA standard dilution series) Quantifiler® Duo Dilution Buffer tube.

† The required number of tubes with the Quantifiler® Duo Primer Mix depends on the number of reactions and the fill level of the tubes. The instrument aspirates 190 µL at a time. In order for the instrument to detect reagent in a tube, the tube must contain a minimum of 240 µL (190 µL plus a 50-µL dead volume). If the volume of reagent in the first tube (position 9) falls below 240 µL, the instrument looks for a sufficient volume in the position 10 tube, then in the position 11 tube. If the tubes are not available or the instrument does not detect sufficient volume, the instrument pauses the run and displays the error message "Not enough liquid."

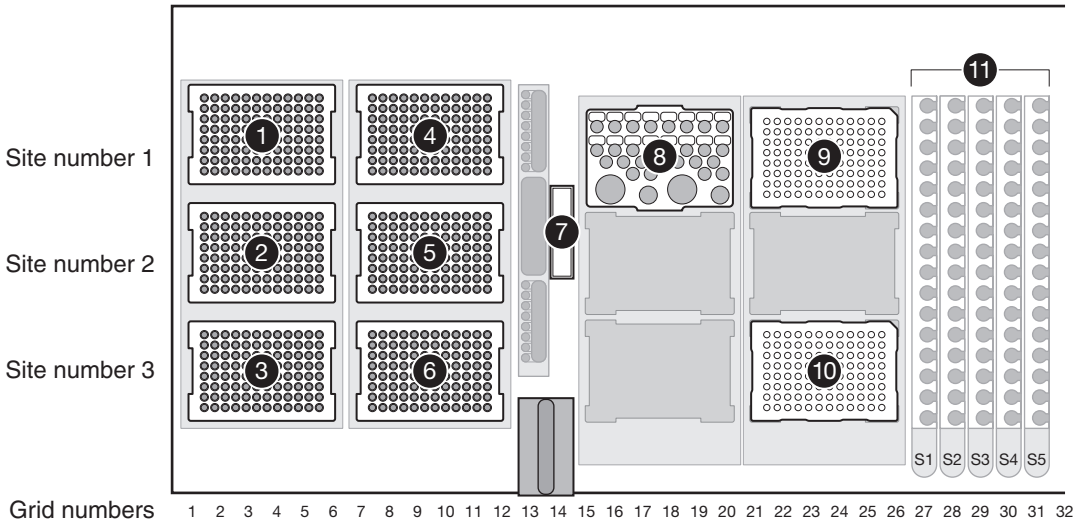
Set up the samples, reagents, and plasticware on the worktable

Set up the samples, reagents, and plasticware as described in Chapter 3 of the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide* and shown below in Table 4.



Note: The TE buffer trough (7 in Table 4 below) is not used for Quantifiler Duo kit runs. Dilution buffer is provided with the kit and is placed in the reagent block.

Table 4 Tecan Freedom EVO® workstation (worktable) layout for a qPCR setup run



Note: Locations for DNA samples in both a plate and tubes are shown, but only one type of plasticware for extracted DNA can be placed on the workstation for a given run.

1–2. 200-µL disposable pipette tips (DiTis)

3–6. 50-µL DiTis

7. **Not used with the Quantifiler® Duo kit:** Trough for T₁₀E_{0.1} buffer (used with Quantifiler® Human and Human Male DNA Quantification Kits only)

8. Chilled qPCR Reagent Block

9. MicroAmp® Optical 96-Well Reaction Plate (qPCR reaction plate) with 96-well metal plate adapter

10. MicroAmp® Optical 96-Well Reaction Plate (if extracted DNA samples are in a plate) with 96-well metal plate adapter

11. Tube racks S1 through S5 for DNA sample tubes (if extracted DNA samples are in tubes)

Run automated qPCR reaction setup

! **IMPORTANT!** For all Quantifiler® kits, you can save time by creating a qPCR/STR Sample file to import sample information into the qPCR/STR system software. If you enter sample information manually for qPCR reaction setup, the information is not retained, and you will need to manually re-enter the information for STR PCR reaction setup. Refer to Chapter 2 of the *HID EVOLution™ – qPCR/STR Setup System Getting Started Guide* for more information on qPCR/STR Sample files.

To run a script, follow the instructions in Chapter 4 of the *HID EVOLution™ – qPCR/STR Setup System Getting Started Guide* using the appropriate Quantifiler® Duo script from the following table:

Freedom EVOWare® software script selection for qPCR reaction setup			
If you are using the kit(s)...	and the sample DNA is in...	...use the script	Maximum number of extracted DNA samples per run
Quantifiler® Duo Kit	a 96-well plate	QuantifilerDuo_plate.esc	80
	1.5-mL microcentrifuge tubes	QuantifilerDuo_tubes.esc	80

Perform qPCR amplification and review results

Convert and transfer the 7500 setup file

! **IMPORTANT!** After qPCR Reaction setup (Quantifiler Duo kit only), you must use the HID EVOLution™ - Quantifiler® Duo Converter Software to convert the 7500 setup file (ReactionPlate1.txt or <barcode>.txt) generated during the qPCR reaction setup run. This creates an importable plate record containing run parameters (including detectors) needed to run the Quantifiler Duo kit assay on the 7500 instrument.

1. On the qPCR/STR system, navigate to the C:\HIDEVOLution_qPCRSTRfiles folder, then locate the file (ReactionPlate1.txt or <barcode>.txt).
2. (Optional) Rename the file.
3. Copy and transfer the ReactionPlate1.txt (or <barcode>.txt) file to a location where it can be accessed by the HID EVOLution™ - Quantifiler® Duo Converter Software.

4. Convert the file:
 - a. Start the Converter.
 - b. Select **Convert Quantifiler Duo 7500 Setup file**, then follow the prompts in the software.

 **Note:** Refer to the *HID EVolution™ - qPCR/STR Setup System*, *HID EVolution™ - Combination System*, *Quantifiler® Duo Converter Manual* (Tecan Document ID 396042) for details.
5. Copy and transfer the converted 7500 setup file to a location where it can be accessed by the SDS software computer.

Import the converted 7500 setup file and perform qPCR

1. Start the 7500 Real-Time PCR System and SDS Software:
 - a. Start the computer.
 - b. Power on the 7500 instrument.
 - c. Start the SDS Software.

 **Note:** For more information on starting the 7500 Real-Time PCR System and software, refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*.
2. Load the qPCR reaction plate into the 7500 instrument (refer to the *Quantifiler® Duo DNA Quantification Kit User's Guide* for details.)
3. Import the converted 7500 setup file into the Applied Biosystems 7500 SDS Software plate document for use during the quantitation run as follows:
 - a. In the SDS software, select **File ▶ New** to create a new Absolute Quantification (Standard Curve) plate document, then in the new document wizard select **Finish**.
 - b. With a blank plate document open, select **File ▶ Import Sample Setup**.
 - c. Browse to locate the converted 7500 setup file, select the file, then click **Open**.
 - d. In the plate document, select the **Instrument** tab, then:
 - Change the Sample Volume to **25 (µL)**
 - Select the **9600 Emulation** box

 **Note:** The thermal cycler protocol validated for use with the Quantifiler® Duo kit includes a hold step at 50 °C for 2 minutes.
 - e. Omit any blank wells.

4. Save the plate document:
 - a. Select **File ▶ Save As**.
 - b. In the Save as dialog box:
 - Enter a file name in the File name field. Do not add an extension to the file name.
 - Select **SDS Document (*.sds)** from the Save as type drop-down list.
 - Click **Save**.
5. Select the **Instrument** tab, then click **Start**.

Analyze and review the qPCR results

1. In the SDS software, click **Analyze**.
 2. Review the standard curve to ensure that results are within the typical range (refer to the *Quantifiler® Duo DNA Quantification Kit User's Manual*). If necessary, omit standard outliers, then click **Analyze** to recalculate the standard curve.
 3. Review the quantitation data and make the necessary determinations:
 - a. Review the quantitation data, including the IPC C_T and the ratio of male to female DNA, and consider the original condition of the sample.
 - b. For each sample, determine if:
 - The sample requires additional processing before STR PCR reaction setup.
 - You will proceed with autosomal STR or Y STR reaction setup.
-  **IMPORTANT!** The qPCR/STR system sets up STR PCR reactions with one AmpF Φ STR® kit per run. We recommend that you view your qPCR results in the SDS software, then determine how you will batch samples for amplification with the appropriate AmpF Φ STR kit.
-  **Note:** After you import the qPCR results to the qPCR/STR system software, you can adjust the target input amount in the Sample Normalization Adjustment window.
-  **Note:** Refer to “Interpretation of Results” in the *Quantifiler® Duo DNA Quantification Kit User's Guide* for details.

Export and convert the 7500 results files



Note: During STR PCR reaction setup, you import the converted 7500 results files to the qPCR/STR system. The EVOware uses the converted 7500 results file to normalize the DNA input amount for STR PCR reaction setup on the qPCR/STR system.

When you are done analyzing the results in the SDS software and have determined the appropriate AmpFSTR® kit for use with each sample, export the 7500 results file(s), convert the file(s), then copy them to the qPCR/STR system computer:

1. In the SDS software, select **File ▶ Export ▶ Results**.
2. In Save as type, select Results Export Files (*.csv), then click **Save**.
3. Use the Converter to create two results files (a human file and a male file).



Note: Refer to the *HID EVolution™ - qPCR/STR Setup System*, *HID EVolution™ - Combination System*, *Quantifiler® Duo Converter Manual* (Tecan Document ID 396042) for details.

4. Copy the converted results files to the computer where the Freedom EVOware® software is installed, or to a server that can be accessed from both systems.

STR PCR reaction setup

When performing STR PCR reaction setup using samples quantified with the Quantifiler Duo kit:

1. Prepare STR PCR reagents and labware as described in Chapter 6 of the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide*.
2. Run STR PCR setup as described in Chapter 7 of the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide*, except that you “[Import the converted 7500 results files](#)” as described below.

Import the converted 7500 results files

-  **IMPORTANT!** The qPCR/STR system sets up STR PCR reactions with one AmpF Φ STR® kit per run. We recommend that you view your qPCR results in the SDS software, then determine how you will batch samples for amplification with the appropriate AmpF Φ STR kit.
1. When you are prompted to import the 7500 results, import either the converted human or male 7500 results files based on the AmpF Φ STR® kit that you selected for use with the samples.
 2. Review the information in the Sample Normalization Adjustment window:
 - a. If necessary, adjust the Req. Amt[ng] to increase or decrease the target DNA input amount added during reaction setup.
 - b. If you want to include samples for STR setup that are not automatically selected:
 - If necessary, adjust the Req. Amt[ng] to increase or decrease the target DNA input amount added during reaction setup.
 - Select the **Process** check box.



Note: For samples with undetermined quantification results in the imported 7500 results file(s), the system adds the maximum volume of DNA sample per reaction. You cannot increase or decrease the volume of DNA sample added to the reaction by changing the value in the Req. Amt.[ng] column.

Section 2 Validation of Quantifiler® Duo Kit on HID EVolution™ – qPCR/STR Setup System

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Overview

The Quantifiler® Duo DNA Quantification Kit (Quantifiler® Duo kit) is now supported on the HID EVOLution™ – qPCR/STR Setup System (qPCR/STR system).

This section describes the validation and verification studies of quantitative PCR (qPCR) setup on the qPCR/STR system using the new Quantifiler Duo scripts and the Quantifiler Duo Converter Software v1.0 that support the Quantifiler® Duo kit.

The following studies were performed:

- Precision and reproducibility studies to evaluate the ability to reproducibly set up Quantifiler Duo reactions using the Quantifiler Duo scripts (EVOware® software v1.4 and v2.1)
- Contamination studies to evaluate the potential for cross-contamination (EVOware® software v1.4 and v2.1)
- Verification studies to evaluate that all files converted using the Quantifiler Duo Converter are compatible with the 7500 Software v1.2.3 and the HID EVOLution system (normalization and STR setup) and that all sample information is complete and accurate (EVOware® software v1.4)

Materials and methods

Materials

Component	Description
Converter	HID EVolution™ Quantifiler® Duo Converter v1.0.0.0
qPCR/STR system	- Freedom EVO® 150 robotic workstation 4-channel LiHa - Freedom EVOware® software [see “Experiments and results: Freedom EVOware® Software v1.4” on page 21 or “Experiments and results: Freedom EVOware® Software v2.1” on page 28 for studies performed on software versions]: - v1.4 SP1, Tecan HID EVolution driver 1.0.0.24, and Tecan Sample Oriented EVOware Limited Edition SP1 - or - v2.1, Tecan HID EVolution driver 1.0.0.25 SP4, Tecan Sample Oriented EVOware 1.2 SP1 - Windows XP Professional operating system - New qPCR setup scripts: - QuantifilerDuo_plate - QuantifilerDuo_tubes
Instruments and software	3130xl Genetic Analyzer Data Collection Software v3.0 - GeneMapper® ID-X Software version 1.0 - GeneAmp® PCR System 9700 Thermal Cycler gold-plated silver 96-well block 7500 Real-Time PCR System with SDS Software v 1.2.3
Consumables	50 µL and 200 µL tips (Tecan Group Ltd.) - TE buffer (Teknova, Hollister, CA)
Chemistry Kits	Quantifiler® Duo DNA Quantification Kit (PN 4387746/Lot Number 0905008)
	AmpFSTR® Identifiler® PCR Amplification Kit (PN 4322288/Lot Number 0809108)
	AmpFSTR® Yfiler® PCR Amplification Kit (PN 4359513/Lot Number 0904046)
	AmpFSTR® MiniFiler™ PCR Amplification Kit (PN 4373872/Lot Number 0904019)
Sample DNA	1 female sample (anonymous donor, Serological Research Institute, S223, L/N 0909240)
	1 male sample (Biochain, Hayward, CA)

Extracted DNA sample plate and tubes set up

The DNA concentrations in one anonymous female (Serological Research Institute) and one commercial male genomic DNA sample (Biochain Institute) were determined using the Quantifiler® Duo DNA Quantification Kit by manual setup and in triplicate.

The samples were then serially diluted manually to obtain concentrations of 50, 10, 5, 2, 1, 0.5, 0.1, and 0.025 ng/μL.

One sample plate containing extracted DNA (source plate) and one set of 1.5 mL tubes containing extracted DNA (source tubes) were prepared manually with 2 to 4 replicates for each concentration and TE buffer blanks (Figures 2 and 3).

Figure 2 Sample plate set up with extracted DNA (source plate)

	Female	Male										
	1	2	3	4	5	6	7	8	9	10	11	12
A	50 ng/uL	TE Blank	5 ng/uL	TE Blank	1 ng/uL	TE Blank	TE Blank	50 ng/uL	1 ng/uL	0.1 ng/uL		
B	TE Blank	50 ng/uL	TE Blank	5 ng/uL	TE Blank	1 ng/uL	TE Blank	50 ng/uL	1 ng/uL	0.1 ng/uL		
C	50 ng/uL	TE Blank	5 ng/uL	TE Blank	1 ng/uL	TE Blank	TE Blank	10 ng/uL	TE Blank	0.1 ng/uL		
D	TE Blank	50 ng/uL	TE Blank	5 ng/uL	TE Blank	1 ng/uL	TE Blank	10 ng/uL	TE Blank	0.1 ng/uL		
E	10 ng/uL	TE Blank	2 ng/uL	TE Blank	0.5 ng/uL	TE Blank	TE Blank	5 ng/uL	0.5 ng/uL	0.025 ng/uL		
F	TE Blank	10 ng/uL	TE Blank	2 ng/uL	TE Blank	0.5 ng/uL	TE Blank	5 ng/uL	0.5 ng/uL	0.025 ng/uL		
G	10 ng/uL	TE Blank	2 ng/uL	TE Blank	0.5 ng/uL	TE Blank	TE Blank	2 ng/uL	TE Blank	0.025 ng/uL		
H	TE Blank	10 ng/uL	TE Blank	2 ng/uL	TE Blank	0.5 ng/uL	TE Blank	2 ng/uL	TE Blank	0.025 ng/uL		

Figure 3 Sample tubes (1.5 mL) set up with extracted DNA (source tubes)

	Female	Male			
	1	2	3	4	5
1	50 ng/uL	TE Blank	1 ng/uL	TE Blank	1 ng/uL
2	TE Blank	5 ng/uL	TE Blank	TE Blank	1 ng/uL
3	50 ng/uL	TE Blank	1 ng/uL	TE Blank	0.5 ng/uL
4	TE Blank	5 ng/uL	TE Blank	TE Blank	0.5 ng/uL
5	10 ng/uL	TE Blank	0.5 ng/uL	TE Blank	0.1 ng/uL
6	TE Blank	2 ng/uL	TE Blank	TE Blank	0.1 ng/uL
7	10 ng/uL	TE Blank	0.5 ng/uL	TE Blank	0.025 ng/uL
8	TE Blank	2 ng/uL	TE Blank	TE Blank	0.025 ng/uL
9	TE Blank	5 ng/uL	TE Blank	50 ng/uL	TE Blank
10	50 ng/uL	TE Blank	1 ng/uL	50 ng/uL	TE Blank
11	TE Blank	5 ng/uL	TE Blank	10 ng/uL	TE Blank
12	50 ng/uL	TE Blank	1 ng/uL	10 ng/uL	TE Blank
13	TE Blank	2 ng/uL	TE Blank	5 ng/uL	TE Blank
14	10 ng/uL	TE Blank	0.5 ng/uL	5 ng/uL	TE Blank
15	TE Blank	2 ng/uL	TE Blank	2 ng/uL	0.025 ng/uL
16	10 ng/uL	TE Blank	0.5 ng/uL	2 ng/uL	0.025 ng/uL

qPCR reaction plate setup

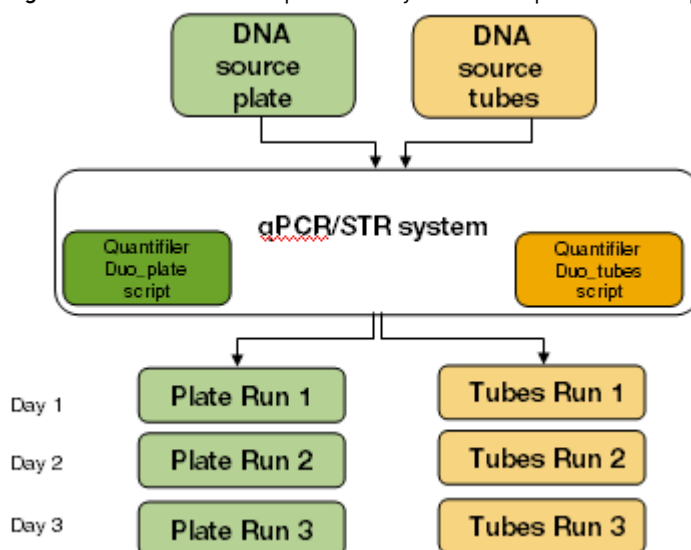
The following scripts, worktable layouts, and Quantifiler Duo reagent block configuration were used.

System	Setup	Script	Worktable layout	Reagent block configuration
qPCR/STR system	qPCR	- QuantifilerDuo_plate - QuantifilerDuo_tubes	See Table 4 on page 10	See Table 3 on page 9

The source plate ([Figure 2 on page 19](#)) and the source tubes ([Figure 3 on page 19](#)) were then run on each system on three consecutive days to create six qPCR reaction plates per system ([Figure 4](#)).

During each qPCR reaction setup run, a fresh standard dilution series of the Quantifiler Duo DNA Standard was prepared on the qPCR/STR system.

Figure 4 Precision and reproducibility studies – qPCR reaction plate setup runs



Studies

Precision and reproducibility studies

On three consecutive days, the two qPCR reaction plates generated from each qPCR setup run (Figure 4 on page 20) were run on the 7500 Real-Time PCR system (six runs total per system). Standard curve slope, R^2 , and sample DNA concentration ranges were evaluated for the Ribonuclease P RNA Component H1 target (RPPH1, total human) and Sex-determining Region Y target (SRY, male).

Contamination studies

C_T values from the precision and reproducibility studies were evaluated. Female samples with C_T values <40 for the Quantifiler Duo Male target and TE buffer blanks with C_T values <40 were manually set up for amplification using the MiniFiler™ PCR Amplification Kit or the Yfiler® PCR Amplification Kit. STR profiles were evaluated in the GeneMapper ID-X software.

Normalization table verification studies

The 7500 results files generated from Plate runs 1, 2, and 3 and Tubes runs 1, 2, and 3 from each system were processed with the Quantifiler Duo Converter to generate human and male 7500 results files. The converted human and male results files from each system were imported into the appropriate Quantifiler Duo script. The dilution schemes (shown in the script Sample Normalization Adjustment window) that the scripts generated for each sample were evaluated.

Experiments and results: Freedom EV0ware® Software v1.4

Precision and reproducibility studies

Experiment

On three consecutive days, the two qPCR reaction plates generated from each qPCR setup run (Figure 4 on page 20) were run on the 7500 Real-Time PCR system (six runs total). Standard curve slope, R^2 , and sample DNA concentration values were evaluated for the Ribonuclease P RNA Component H1 target (RPPH1, total human) and Sex-determining Region Y target (SRY, male)

Slope and R^2

Observed slope and R^2 values are summarized in Table 5. The slope and R^2 values were within the typical range of slope values observed during the development and validation of the Quantifiler® Duo kit (slope: -3.0 to -3.6 and R^2 : >0.98, respectively).

Table 5 Slope and R^2 values for standard curves

Run	Source plate				Source tubes			
	Duo Human		Duo Male		Duo Human		Duo Male	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
1	-3.02	0.997	-3.18	0.998	-3.28	0.998	-3.42	0.995
2	-3.19	0.998	-3.22	0.996	-3.24	0.997	-3.37	0.994
3	-3.24	0.998	-3.39	0.992	-3.29	0.998	-3.39	0.997
Avg	-3.15	0.998	-3.26	0.995	-3.27	0.998	-3.39	0.995

DNA concentration The range of variation in the quantification results is:

- Comparable to the results seen during developmental validation (reported in the *Quantifiler® Duo PCR Amplification User Guide*) at all tested concentrations of DNA (among replicates and across three runs)
- Similar for source plate and source tubes

DNA concentrations from the three runs for the source plate and tubes are summarized in [Figures 5 through 8](#) on [pages 22 and 24](#).

The standard deviation values and concentration ranges are summarized in [Table 6](#) on [page 25](#) and [Table 7](#) on [page 26](#).

Figure 5 Quantification results obtained from the RPPH1 target (total human) qPCR reaction plates prepared using QuantiflerDuo_plate script from DNA source plate

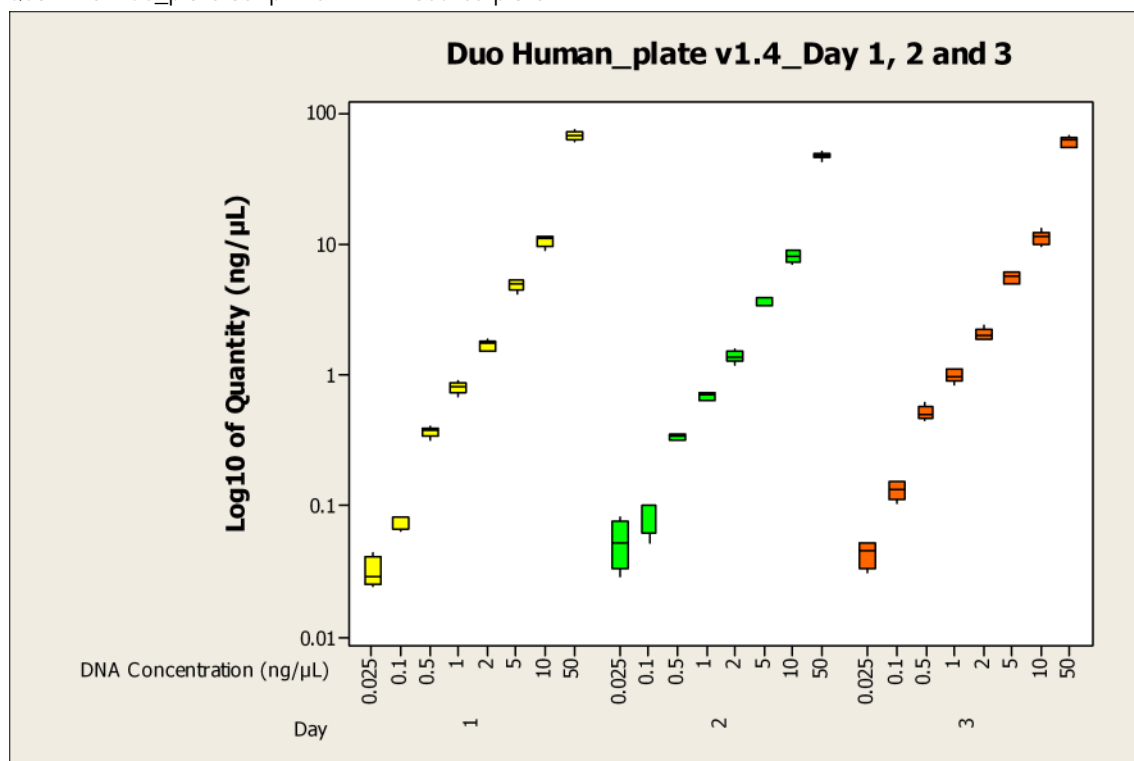


Figure 6 Quantification results obtained from the SRY target (male) in the qPCR reaction plates prepared using QuantiflerDuo_plate script from DNA source plate

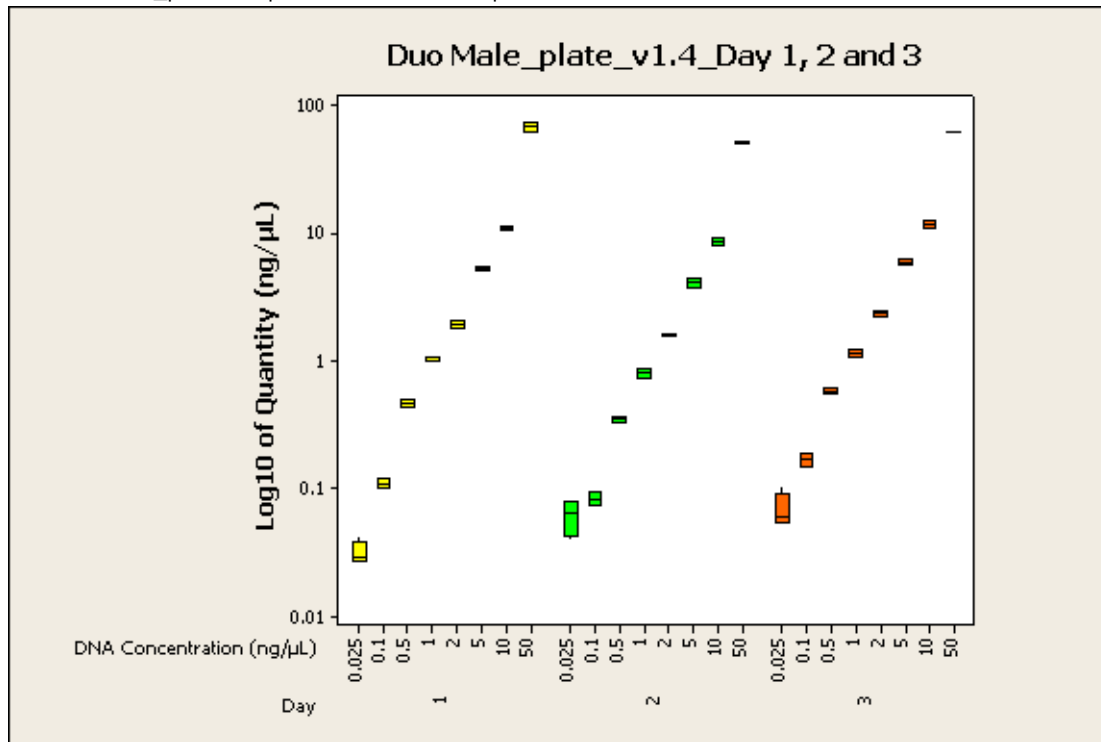


Figure 7 Quantification results obtained from the RPPH1 target (total human) in the qPCR reaction plates prepared using QuantiflerDuo_tubes script from DNA source tubes

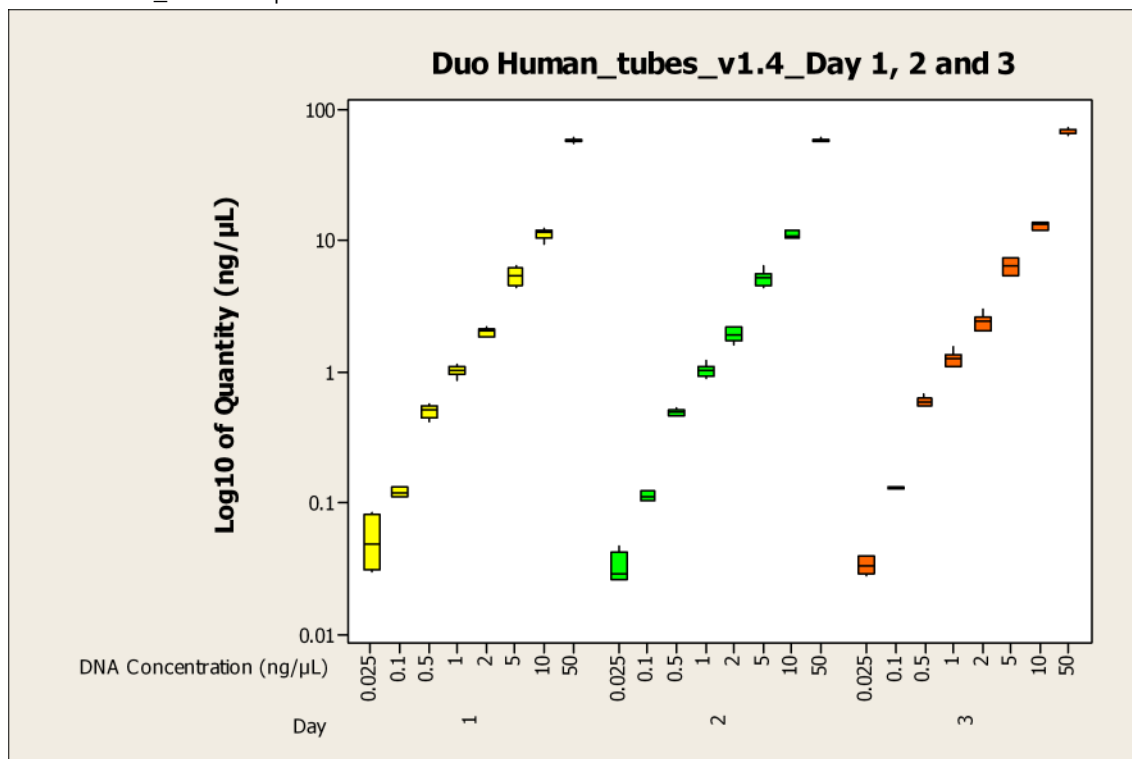


Figure 8 Quantification results obtained from the SRY target (male) in the qPCR reaction plates prepared using QuantiflerDuo_tubes script from DNA source tubes

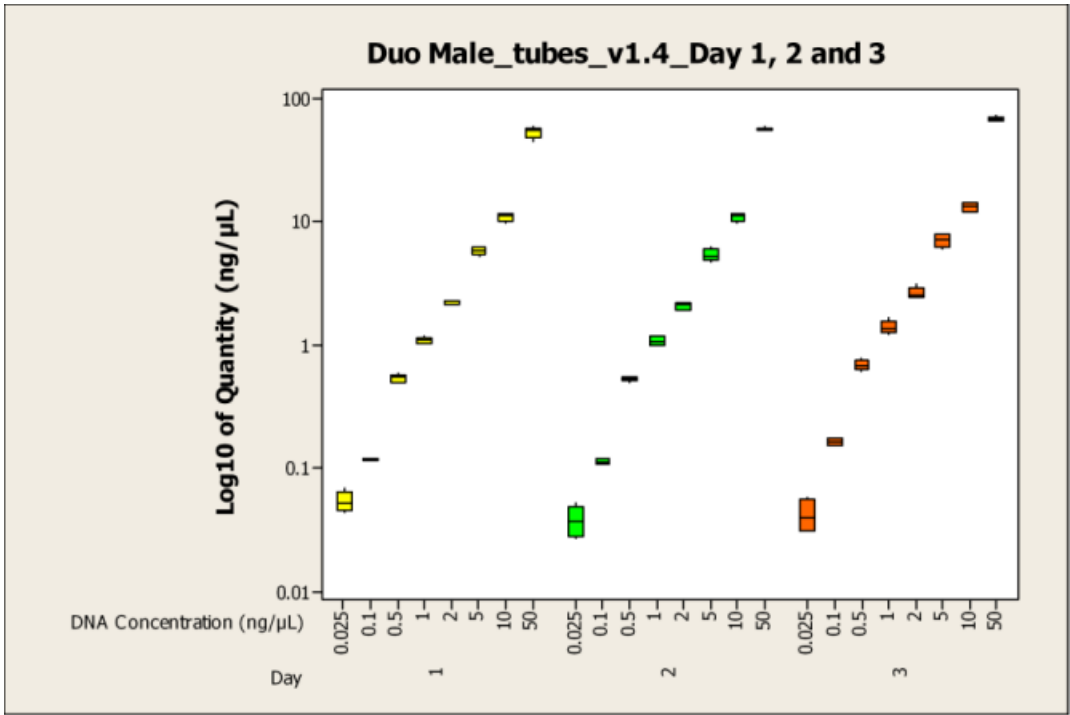


Table 6 RPPH1 target (human) DNA standard deviations and concentrations

Male Sample	Standard Deviations							
Expected (ng/μL)	n (for Plate)	Plate 1	Plate 2	Plate 3	n (for Tubes)	Tubes 1	Tubes 2	Tubes 3
0.025	4	0.009	0.022	0.010	4	0.028	0.009	0.005
0.1	4	0.009	0.024	0.021	2‡	N/A‡	N/A‡	N/A‡
0.5	4	0.018	0.008	0.052	4	0.025	0.018	0.052
1	4	0.033	0.023	0.069	4	0.066	0.123	0.147
2	4	0.079	0.133	0.162	4	0.048	0.192	0.255
5	4	0.351	0.235	0.331	4	0.595	0.574	0.656
10	4	0.311	0.843	0.849	4	0.598	0.541	0.346
50	4	3.949	1.505	1.488	4	0.888	1.386	3.261
Female Sample	Concentration (ng/μL)							
(Expected ng/μL)	n (for Plate)	Plate 1	Plate 2	Plate 3	n (for Tubes)	Tubes 1	Tubes 2	Tubes 3
0.5	2	0.35; 0.32	0.31; 0.32	0.45; 0.46	2	0.46; 0.42	0.46; 0.46	0.60; 0.55
1	2	0.76; 0.67	0.64; 0.63	0.92; 0.85	2	0.99; 0.86	0.98; 0.89	1.08; 1.12
2	2	1.53; 1.54	1.19; 1.46	1.85; 1.87	2	1.82; 1.82	1.63; 1.84	2.05; 2.04
5	2	4.12; 4.50	3.44; 3.86	4.88; 4.94	2	4.37; 4.55	4.66; 4.38	5.43; 5.45
10	2	8.98; 9.92	7.05; 7.70	9.63; 10.15	2	9.48; 10.74	10.59; 10.48	12.32; 11.85
50	2	59.86; 64.48	42.85; 46.47	53.61; 54.78	2	54.92; 58.79	57.46; 58.41	64.06; 65.04

‡ Due to the number of wells on the qPCR reaction plate, only two samples were run. Standard deviation not calculated.

Table 7 SRY target (male) DNA standard deviations

Male Sample	Standard Deviations							
Expected (ng/μL)	n (for Plate)	Plate 1	Plate 2	Plate 3	n (for Tubes)	Tubes 1	Tubes 2	Tubes 3
0.025	4	0.007	0.019	0.023	4	0.011	0.011	0.013
0.1	4	0.010	0.012	0.022	2‡	N/A‡	N/A‡	N/A‡
0.5	4	0.029	0.016	0.031	4	0.039	0.021	0.066
1	4	0.037	0.063	0.066	4	0.059	0.091	0.180
2	4	0.141	0.041	0.107	4	0.090	0.133	0.289
5	4	0.263	0.399	0.448	4	0.425	0.624	0.873
10	4	0.275	0.601	0.958	4	0.738	0.737	1.188
50	4	5.385	0.948	0.615	4	5.619	1.597	2.549

‡ Due to the number of wells on the qPCR reaction plate, only two samples were run. Standard deviation not calculated.

Contamination study

Experiment C_T values from the precision and reproducibility studies were evaluated.

Results The results of the contamination study are summarized in [Table 8](#).
Five TE buffer blanks exhibited C_T values <40 and were manually set up for amplification using the MiniFiler™ PCR Amplification Kit. None of these 5 TE buffer blanks exhibited STR profiles.
One female sample exhibited a C_T value of 37.97 for the Duo Male target and was manually set up for amplification using the Yfiler® and MiniFiler™ PCR Amplification kits. However, this sample did not yield a Y-STR profile when amplified using the Yfiler PCR Amplification Kit. Similarly, the autosomal STR profile obtained using the MiniFiler kit did not indicate the presence of a mixture. Therefore, the lower C_T values are attributed to nonspecific amplification.

Table 8 TE buffer blank C_T and STR results (N/A = samples not set up for amplification)

Run	Total # Samples	Female samples with C _T <40 for male detector	STR Results		Total # TE blanks	TE Blanks Exhibiting C _T <40 for male and/or human detector	STR Results
			Yfiler Kit	MiniFiler Kit			MiniFiler Kit
Plate 1	44	None	N/A	N/A	36	None	N/A
Plate 2	44	None	N/A	N/A	36	TEBlank 5 (well A2): Duo Human C _T = 37.03	No STR profile
Plate 3	44	None	N/A	N/A	36	TEBlank 17 (well B5): Duo Male C _T = 37.65	No STR profile
						TEBlank 20 (well H5): Duo Human C _T = 39.20	No STR profile
Tube 1	42	None	N/A	N/A	38	None	N/A
Tube 2	42	None	N/A	N/A	38	TEBlank 3 (position 6, carrier 1): Duo Male C _T = 38.20	No STR profile
Tube 3	42	2 ng/μL female sample (position 29 in carrier 2) exhibited C _T value of 37.97 for male target	No Male STR profile was detected with Yfiler	No indication of mixture STR profile	38	TEBlank 15 (position 30, carrier 2): Duo Human C _T = 36.85	No STR profile

Verification of normalization setup

Experiment The 7500 results files generated from Plate runs 1, 2, and 3 and Tubes runs 1, 2, and 3 from each system were processed with the Quantifiler Duo converter to generate human and male 7500 results files. The converted human and male results files from each system were imported into the appropriate Quantifiler Duo script. The dilution schemes (shown in the script Sample Normalization Adjustment window) that the scripts generated for each sample were evaluated.

Normalization values The dilution schemes (shown in the script Sample Normalization Adjustment window) that the scripts generated for each sample were evaluated. Total dilution (T Dil.) values for each concentration of human and male DNA were examined to ensure that the calculated dilutions were appropriate for each concentration. Refer to the *HID EVolution™ – qPCR/STR Setup System Getting Started Guide* for information on the dilution protocols used by the qPCR/STR system software.

Experiments and results: Freedom EVOware® Software v2.1

Precision and reproducibility study

Experiment The source plate ([Figure 2 on page 19](#)) and source tubes ([Figure 3 on page 19](#)) were run using the QuantifilerDuo_plate script. The two qPCR reaction plates were run on the 7500 Real-Time PCR system. Sample DNA concentration were evaluated.

DNA concentration The range of variation in the quantification results is:

- Comparable to the results seen during developmental validation (reported in the *Quantifiler® Duo PCR Amplification User Guide*) at all tested concentrations of DNA.
- Similar for source plate and source tubes.

DNA concentrations are summarized in [Figures 9 through 12 on pages 29 and page 30](#).

The standard deviation values and concentration ranges for human and male DNA quantity values are summarized in [Tables 9 and 10 on page 31](#).

Figure 9 EVOware® software v2.1: Quantification results obtained from the RPPH1 target (total human) qPCR reaction plates prepared using QuantiflerDuo_plate script from DNA source plate

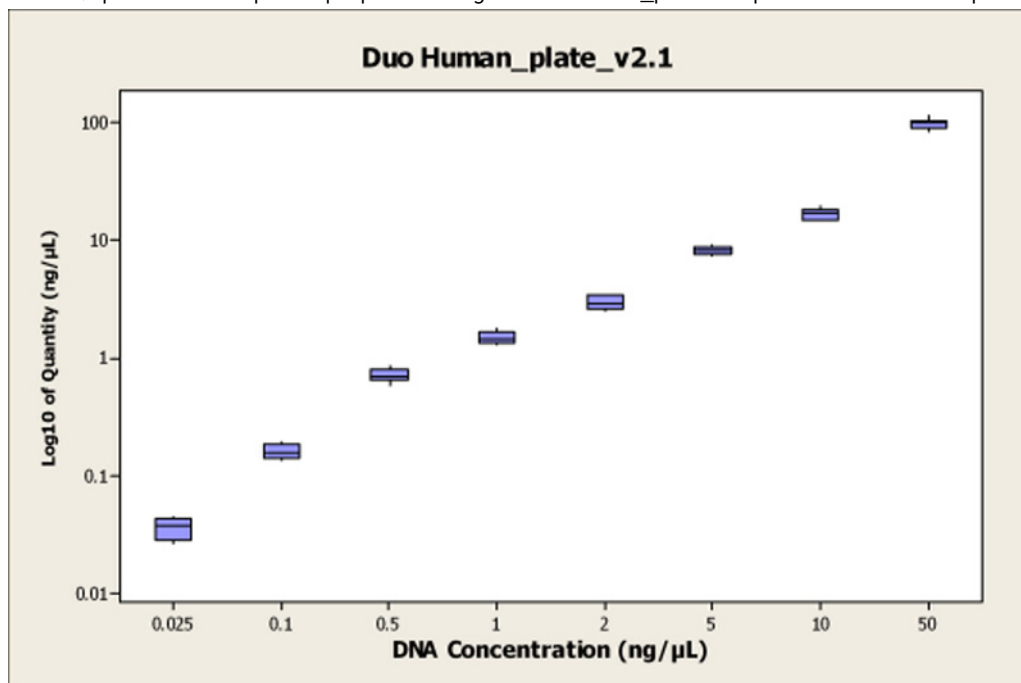


Figure 10 EVOware® software v2.1: Quantification results obtained from the SRY target (male) in the qPCR reaction plates prepared using QuantiflerDuo_plate script from DNA source plate

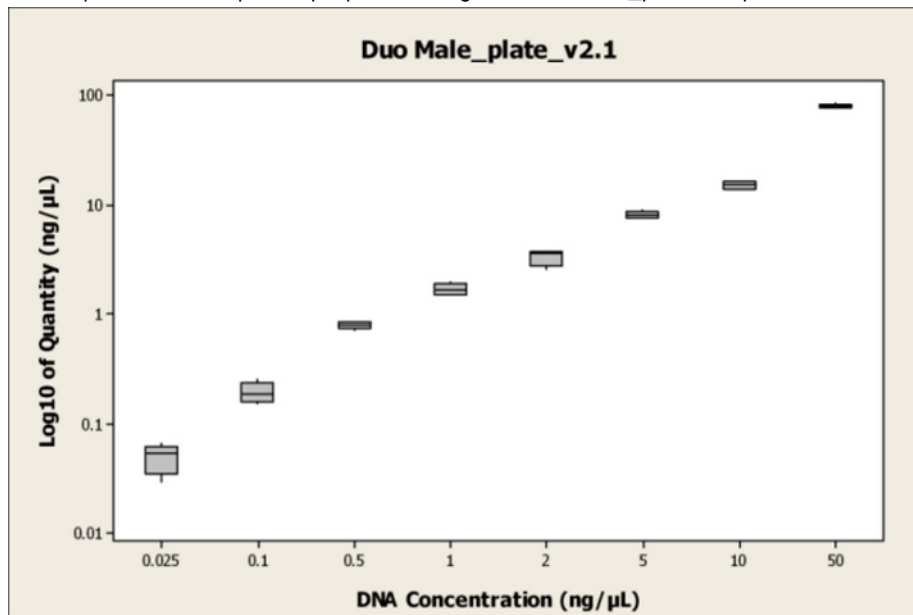


Figure 11 EVOware® software v2.1: Quantification results obtained from the RPPH1 target (total human) in the qPCR reaction plates prepared using QuantiflerDuo_tubes script from DNA source tubes

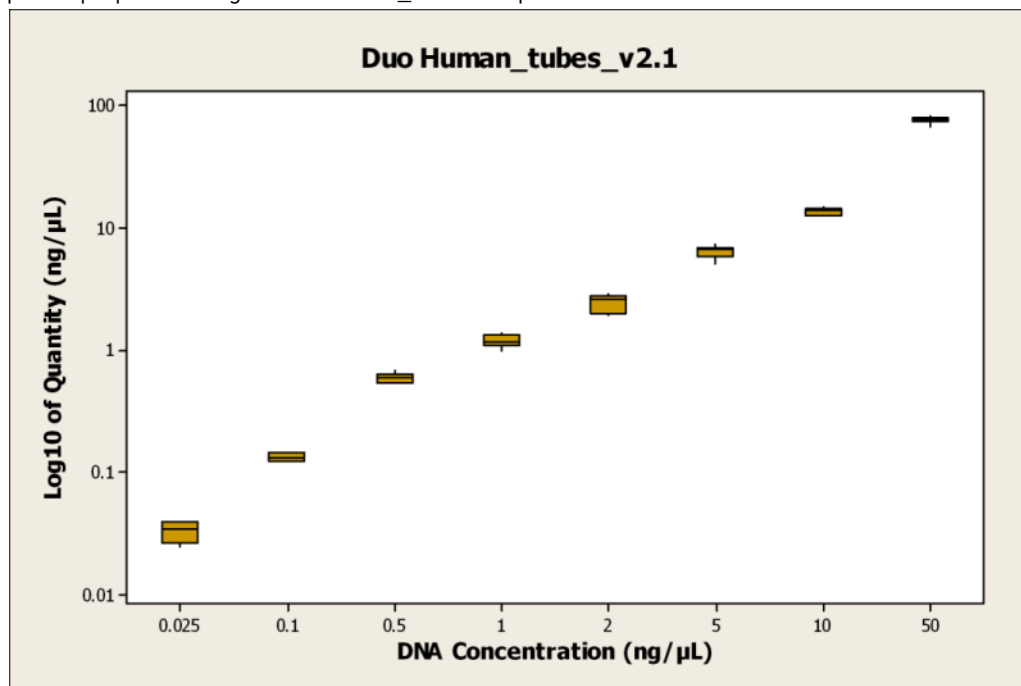


Figure 12 EVOware® software v2.1: Quantification results obtained from the SRY target (male) in the qPCR reaction plates prepared using QuantiflerDuo_tubes script from DNA source tubes

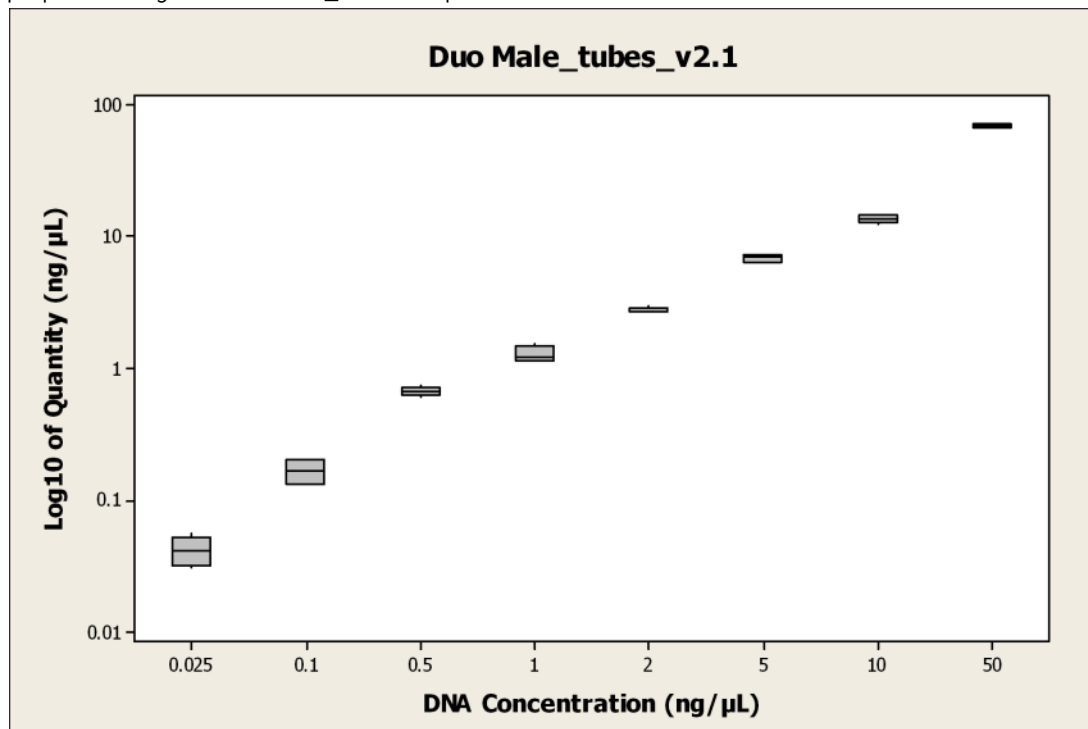


Table 9 RPPH1 target (human) DNA standard deviations and concentrations

Male Sample	Standard Deviations			
Expected (ng/ μL)	n (for Plate)	Plate	n (for Tubes)	Tubes
0.025	4	0.008	4	0.007
0.1	4	0.024	2 [‡]	N/A [‡]
0.5	4	0.080	4	0.054
1	4	0.161	4	0.183
2	4	0.354	4	0.120
5	4	0.450	4	0.324
10	4	0.992	4	0.319
50	4	7.082	4	5.746
Female Sample	Concentration (ng/μL)			
Expected (ng/ μL)	n (for Plate)	Plate	n (for Tubes)	Tubes
0.5	2	0.59; 0.71	2	0.61; 0.54
1	2	1.29; 1.35	2	1.10; 1.18
2	2	2.52; 2.59	2	2.05; 1.93
5	2	7.73; 7.27	2	6.12; 5.17
10	2	14.70; 14.84	2	12.52; 12.52
50	2	90.35; 83.46	2	79.98; 75.85

[‡] Due to the number of wells on the qPCR reaction plate, only two samples were run. Standard deviation not calculated.

Table 10 SRY target (male) DNA standard deviations and concentrations

Male Sample	Standard Deviations			
Expected (ng/μL)	n (for Plate)	Plate	n (for Tubes)	Tubes
0.025	4	0.015	4	0.011
0.1	4	0.044	2	N/A
0.5	4	0.053	4	0.056
1	4	0.243	4	0.179
2	4	0.512	4	0.130
5	4	0.645	4	0.518
10	4	1.223	4	0.928
50	4	3.328	4	2.727

Contamination study

Experiment C_T values from the precision and reproducibility studies were evaluated. If any female samples exhibited C_T values <40 for the Quantifiler Duo Male target and if any TE buffer blanks exhibited C_T values <40, they were manually set up for amplification using the MiniFiler™ PCR Amplification Kit or the Yfiler® PCR Amplification Kit. STR profiles were evaluated in the GeneMapper ID-X software.

Results The results of contamination study are summarized in [Table 11](#).
One TE buffer blank exhibited C_T value <40 and was manually set up for amplification using the MiniFiler™ PCR Amplification Kit. However, this TE buffer blank did not exhibit an STR profile when amplified using the MiniFiler™ kit. Therefore, the lower C_T value is attributed to nonspecific amplification.

Table 11 TE buffer blank C_T and STR results (N/A = samples not set up for amplification)

Run	# Samp	# of Samples with C_T <40	STR Results		#TE Blank	#TEBlanksExhibiting C_T <40	STR Results
			Yfiler Kit	MiniFiler Kit			MiniFiler Kit
Plate	44	None	N/A	N/A	36	None	N/A
Tube	42	None	N/A	N/A	38	TEBlank24 (position 47, carrier 3): Duo Human Ct is 38.84	No STR profile

Conclusions

The Quantifiler Duo scripts and Quantifiler Duo converter were validated on the HID EVolution™ – qPCR/STR Setup System using the Quantifiler® Duo DNA Quantification Kit:

- Precision and reproducibility studies confirm that the new Quantifiler Duo scripts and Quantifiler Duo converter provide reliable results at different DNA input amounts on Freedom EVOware® Software v1.4 and v2.1.
- Contamination studies demonstrated clean liquid handling capabilities for the qPCR setup on EVOware® Software v1.4 and v2.1.
- Normalization and STR setup studies confirm that the new Quantifiler Duo scripts and Quantifiler Duo converter provide accurate and reliable data handling for normalization and STR setup.
- The stability study confirms that the Quantifiler Duo scripts prepare diluted standards that meet the performance requirements when stored according to the conditions recommended in the *Quantifiler® Duo DNA Quantification Kit User Guide*.

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